Interactions between Methylsulfonyl PCBs and the Glucocorticoid Receptor

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Persistent polychlorinated biphenyl (PCB) metabolites were studied with respect to their interaction with the human glucocorticoid receptor (GR). 3-Methylsulphonyl-2,5,6,2′,4′,5′-hexachlorobiphenyl (3-$MeSO_2$-CB149) was shown to compete with ^{3}H-dexamethasone for binding to the GR, with an IC_{50} (concentration that inhibits 50%) of approximately 1 μM. Using GRAF cells expressing human GR, glucocorticoid responsive element, and a reporter enzyme, we demonstrated that 3-$MeSO_2$-CB149 functionally acts as an antagonist at the GR (IC_{50} = 2.7 μM). In accordance with the receptor binding, the antagonism mainly appeared to be of a competitive nature. When studying the competitive binding of 24 methylsulfonyl PCBs (relative to dexamethasone) to GR from mouse liver cytosol, seven compounds had a higher affinity to GR than 3-$MeSO_2$-CB149. Structure–activity relationship studies indicated that the presence of three chlorine atoms in the *ortho*-position and chlorine and methyl sulfone groups on either end of the molecule (4 and 4′-position) increased the affinity to GR. The relevance of this finding for human health is not known, but PCB methyl sulfones are ubiquitous pollutants present in mother's milk. The results stress the need for studying endocrine disruptors that affect hormonal systems other than sex and thyroidogenic hormones. *Key words*: endocrine disruptor, glucocorticoid receptor, methyl sulphone, PCB, polychlorinated biphenyls, xenobiotic. *Environ Health Perspect* 106:769–772 (1998). [Online 12 November 1998]
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A lot of attention has been focused on the ability of environmental pollutants to affect estrogen homeostasis, e.g., by binding to the estrogen receptor as agonists or antagonists, thereby affecting the sex differentiation of organisms (*1,2*). Little concern has been paid to the ability of pollutants to interfere with other steroid hormone receptors such as the glucocorticoid receptor (GR), although benzpyrene, for example, has been shown to affect the GR density in rats (*3*).

The decreasing populations of gray seals (*Halichoerus grypus*) and harbor seals (*Phoca vitulina*) from the heavily polluted Baltic Sea suffer from a disease syndrome suggested to be caused by an increased exposure to glucocorticoid hormones (hyperadrenocorticism) (*4*). The syndrome is characterized by claw deformations, skin abnormalities, adrenocortical hyperplasias, osteoporosis, uterine lesions, and an impaired immune system. However, the two most abundant pollutants in the Baltic, i.e., polychlorinated biphenyls (PCBs) and dichlorodiphenyl dichloroethylene (DDE), have not been shown to elicit all these effects in any experimental animals. A possible explanation for some of these symptoms is that environmental pollutants may act as glucocorticoid agonists or antagonists, thereby interrupting glucocorticoid homeostasis in the animals.

The present study was undertaken to investigate if environmental pollutants have affinity to the GR or interfere with the glucocorticoid signaling pathway. We used human and mouse GRs for the binding studies and a well-defined cellular system (human GR and GR responsive elements transfected into CHO cells) for effect studies. As prototype chemicals, some methylsulfone metabolites of PCBs and DDE were chosen. Methylsulfonyl PCBs are the third most abundant pollutants in, for example, Scandinavian and Canadian biota (*5*), but are still seldom studied with respect to biological effects. 3-Methylsulfonyl DDE is an adrenocortical toxicant following metabolic activation by a steroid (CYP11B1) hydroxylase (*6*). The effects of the xenobiotics were compared with that of RU486, a drug that acts as a competitive antagonist at the GR. RU486 inhibits cortisol-dependent functions *in vivo* in humans, which, due to the feed-back systems, increases the plasma levels of adrenocorticotropic hormone (ACTH) and cortisol (*7,8*).

Materials and Methods

Reagents and supplies. The used methylsulfonyl PCBs and 3-methylsulfonyl DDE (3-$MeSO_2$-DDE) (see Table 1) were synthesized according to Bergman and Wachtmeister (*9,10*) and Haraguchi et al. (*11*). ^{3}H-Dexamethasone (40 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). Dexamethasone, molybdic acid, bovine serum albumin, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). 1,4-Dithio-DL-threitol (DTT), EDTA, and potassium phosphate were from Fluka (Buchs, Switzerland). RU486 was a kind gift from Roussel Uclaf (Paris, France). Ham's F12 medium, L-glutamine, fetal calf serum (FCS), and gentamicin were purchased from Gibco BRL/Life Technologies (Täby, Sweden), phenol red-free Coon's/F12 medium was prepared by SVA (Uppsala, Sweden), and SRC 3000 serum substitute was purchased from Tissue Culture Services (Botolph Claydon, Buckingham, UK). The chemiluminescence substrate AMPPD was purchased from Tropix (Boston, MA), via Boule Diagnostics (Huddinge, Sweden). The CellTiter 96 Cell Proliferation kit was purchased from Promega via Scandinavian Diagnostics Services (Falkenberg, Sweden). White microtitration plates were obtained from Dynatech Laboratories via In Vitro AB (Stockholm, Sweden), and Costar cell culture plastics were purchased from Life Technologies (Täby, Sweden). All plastics used for binding studies with GR were of polypropylen. The graphs were drawn using the computer program KaleidaGraph (Synergy Software, Reading, PA).

Preparation of GR. Human GR was extracted from insect cells infected with recombinant baculovirus encoding full-length human GR. The cells were homogenized in phosphate buffer (20 mM, pH 8.0) containing 1 mM EDTA, 100 mM KCl, 20 mM MoO_4, 8.8% glycerol, and 2 mM DTT. The homogenate was centrifuged at 8,000*g*, and the resulting GR-containing supernatant was stored at -70°C until used. Mouse liver was homogenized as described above and the homogenate was centrifuged at 100,000*g* for 1 hr. The resulting GR-containing cytosol was stored at -70°C until used.

Competition binding studies with GR. GR (1 nmol, as determined by saturation studies), ^{3}H-dexamethasone (5 nM), and increasing concentrations of DDE- and PCB methyl sulfones (1 nM–30 μM in DMSO) were incubated overnight on ice in the buffer used for extraction of GR (modified to contain 10% glycerol and 1 mM DTT). Free ^{3}H-dexamethasone was separated from

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Table 1. The inhibitory effects of PCB methyl sulfones on competition binding assays using ^{3}H-dexamethasone and mouse liver cytosolic glucocorticoid receptor (GR)

Compound	IC_{25} (μM)	IC_{50} (μM)
4-$MeSO_2$-2,3,6,2′,4′,5′-hexaCB (4-$MeSO_2$-CB149)	5 ± 4	15 ± 6
4-$MeSO_2$-2,5,6,2′,4′-pentaCB (4-$MeSO_2$-CB91)	7 ± 4	17 ± 6
4-$MeSO_2$-2,3,6,2′,3′,4′-hexaCB (4-$MeSO_2$-CB132)	9 ± 5	17 ± 4
4-$MeSO_2$-2,3,6,2′,3′,4′,5′-heptaCB (4-$MeSO_2$-CB174)	6–8	16–18
4-$MeSO_2$-2,3,6,3′,4′-pentaCB (4-$MeSO_2$-CB110)	NI	
4-$MeSO_2$-2,3,6,4′-tetraCB (4-$MeSO_2$-CB64)	NI	
4-$MeSO_2$-2,5,2′,4′-tetraCB (4-$MeSO_2$-CB49)	NI	
4-$MeSO_2$-2,5,2′,5′-tetraCB (4-$MeSO_2$-CB52)	NI	
4-$MeSO_2$-2,5,3′,4′-tetraCB (4-$MeSO_2$-CB70)	NI	
4-$MeSO_2$-2,5,2′,3′,4′-pentaCB (4-$MeSO_2$-CB87)	NI	
4-$MeSO_2$-2,5,2′,4′,5′-pentaCB (4-$MeSO_2$-CB101)	NI	
4-$MeSO_2$-2,5,2′,3′,4′,5′-hexaCB (4-$MeSO_2$-CB141)	NI	
3-$MeSO_2$-2,5,6,2′,3′,4′,5′-heptaCB (3-$MeSO_2$-CB174)	5–10	20–20
3-$MeSO_2$-2,5,6,2′,4′-pentaCB (3-$MeSO_2$-CB91)	12–13	>30
3-$MeSO_2$-2,5,6,2′,3′,4′-hexaCB (3-$MeSO_2$-CB132)	16–18	>30
3-$MeSO_2$-2,5,6,2′,4′,5′-hexaCB (3-$MeSO_2$-CB149)	18–20	>30
3-$MeSO_2$-2,5,6,3′,4′-pentaCB (3-$MeSO_2$-CB110)	NI	
3-$MeSO_2$-2,5,6,4′-tetraCB (3-$MeSO_2$-CB64)	NI	
3-$MeSO_2$-2,5,2′,4′-tetraCB (3-$MeSO_2$-CB49)	NI	
3-$MeSO_2$-2,5,2′,5′-tetraCB (3-$MeSO_2$-CB52)	NI	
3-$MeSO_2$-2,5,3′,4′-tetraCB (3-$MeSO_2$-CB70)	NI	
3-$MeSO_2$-2,5,2′,3′,4′-pentaCB (3-$MeSO_2$-CB87)	NI	
3-$MeSO_2$-2,5,2′,4′,5′-pentaCB (3-$MeSO_2$-CB101)	NI	
3-$MeSO_2$-2,5,2′,3′,4′,5′-hexaCB (3-$MeSO_2$-CB141)	NI	

Abbreviations: IC_{25}, concentration that inhibits 25%; IC_{50}, concentration that inhibits 50%; CB, chlorinated biphenyl; NI, no inhibition, i.e., the compound lacks affinity to the GR at the doses tested. The values shown are mean ± standard deviation (n = 3) or range (n = 2).

GR-bound ^{3}H-dexamethasone by elution through a Sephadex column (QS-2A, Boule Diagnostics AB, Huddinge, Sweden). ^{3}H-dexamethasone bound to GR was measured by liquid scintillation of the protein phase. The dissociation constants (K_d) for the binding of ^{3}H-dexamethasone to human and mouse GR were 5–10 nM.

Cell culture. The GRAF reporter cell line was generated by stable transformation of Chinese hamster ovary cells (CHO K1, ATCC No. CCL 61) with the mammalian expression vector pmT-hGR (*12*) and the glucocorticoid responsive reporter vector MMTV-ALP (*13*). GRAF cells were routinely cultured in Ham's F12 medium supplemented with 10% FCS and 2 μM L-glutamine (in 37°C humidified chambers at 5% CO_2).

Studies of GR-mediated effects in GRAF cells. Before each experiment, the GRAF cells were seeded in 96-well tissue culture plates (approximately 25 × 10^3 cells/well) in phenol red-free Ham's F12 medium supplemented with 10% FCS (stripped with dextran-coated charcoal), 2 μM L-glutamine, and gentamicin (50 μg/ml). On day 2 cells were rinsed with Coon's/F12 medium supplemented with 5% SRC 3000, 2 μM L-glutamine, and gentamicin (50 μg/ml), and were given 100 μl of the same medium supplemented with dexamethasone and xenobiotics (1 nM–10 μM) at various concentrations. In all experiments, the cells were exposed to substances for 48 hr before being harvested and analyzed for effects on gene expression. Triplicate samples of each concentration of compounds were used in the experiments.

Toxicity was assessed by microscopic evaluation of cell morphology and by the CellTiter 96 cell proliferation assay in which the mitochondrial formation of a colored tetrazolium salt is measured spectrophotometrically at 492 nm (*14*). In the assay, absorbance is directly proportional to the number of living cells in each culture.

Assay for human placental alkaline phosphatase. Conditioned medium was analyzed for the relative levels of placental alkaline phosphatase (ALP) reporter protein expressed and secreted from the GRAF reporter cells by a chemiluminescent assay. A 10-μl aliquot of the conditioned cell culture medium was mixed with 200 μl of assay buffer (10 mM diethanolamine, pH 10.0; 1 mM $MgCl_2$; and 0.5 mM AMPPD) in white microtiter plates and incubated for 20 min at 37°C before being transferred to a microplate format luminometer (Luminoskan; Labsystems, Helsinki, Finland). The luminometer was set on integral measurement with 1 sec reading of each well. The ALP activity was expressed in light units (LU), which are directly proportional to the level of ALP secreted from the cells.

Results

The ability of the xenobiotics to compete with ^{3}H-dexamethasone for binding to human GR is shown in Figure 1. 3-$MeSO_2$-CB149 (Fig. 2) almost completely removed dexamethasone from GR at a concentration of 10 μM, with an IC_{50} (concentration that inhibits 50%) of approximately 1 μM. The IC_{50} of unlabeled dexamethasone was approximately 6 nM. Also 3-$MeSO_2$-CB101 affected binding of dexamethasone to GR, although with a relative affinity approximately 10-fold lower than that of 3-$MeSO_2$-CB149. 3-$MeSO_2$-DDE did not bind to human GR.

None of the three xenobiotics activated the GR in GRAF cells (measured as induction

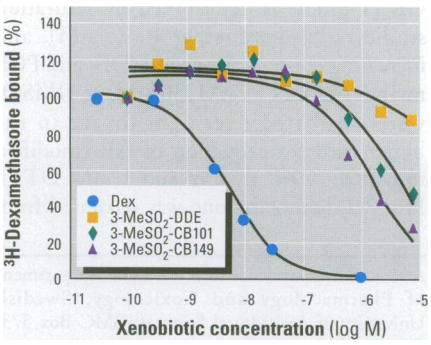

Figure 1. Competition binding studies to the human glucocorticoid receptor (GR). Receptor (1 nmol) was incubated overnight with ^{3}H-dexamethasone (Dex) and either 3-$MeSO_2$-CB149, 3-$MeSO_2$-CB101, 3-$MeSO_2$-DDE, or unlabeled Dex, after which ^{3}H-Dex bound to GR was quantified. Each point represents the mean of duplicate samples.

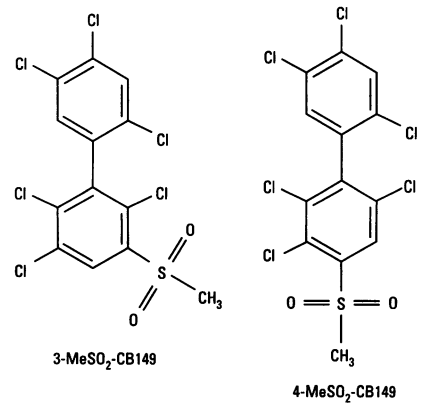

Figure 2. The chemical structures of 3-$MeSO_2$-CB149 and 4-$MeSO_2$-CB149. A methyl sulfone group in the 4 position instead of the 3 position increases the affinity to mouse glucocorticoid receptor (GR). Other important structures for binding to GR seem to be three *ortho*-chlorines in the 2,6,2′-positions and a chlorine in the 4′ position.

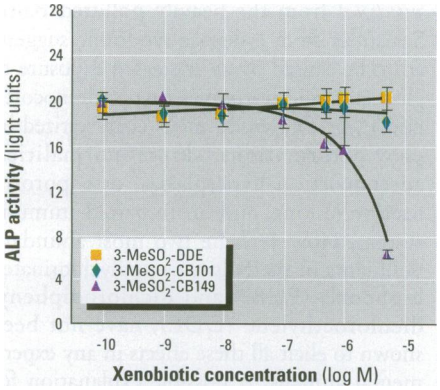

Figure 3. Effect of 3-$MeSO_2$-CB149, 3-$MeSO_2$-CB101, and 3-$MeSO_2$-DDE on the induction of alkaline phosphatase (ALP) by dexamethasone in GRAF cells. The concentration of 10^{-10} M in the graph represents the absence of methyl sulfone, i.e., exposure to 5 nM dexamethasone in the presence of solvent only. No signs of toxicity were observed at the concentrations shown in the figure. Each point represents mean ± standard deviation of triplicate samples.

of; ALP; data not shown). The effective concentration (EC_{50}) for dexamethasone was 4.6 nM. The ability of the xenobiotics to block the dexamethasone-mediated effect was studied next. 3-$MeSO_2$-CB149 completely inhibited dexamethasone-mediated induction of ALP, with an IC_{50} of 2.7 µM (Fig. 3). 3-$MeSO_2$-DDE decreased ALP at 10 µM, but a very steep dose– response curve and severe signs of toxicity at 25 µM make interpretation of these data difficult (data not shown). Finally, 3-$MeSO_2$-CB101 had no effect on the dexamethasone-dependent induction of ALP.

To assess the nature of the inhibitory effect of the xenobiotics on GR-mediated induction of ALP, the effect of the xenobiotics was compared with that of RU486, a competitive antagonist to GR. As shown in Figure 4A, RU486 competitively antagonized the effect of dexamethasone (RU486 dose-dependently increased the EC_{50} of dexamethasone, but did not affect the maximum induction mediated by dexamethasone). 3-$MeSO_2$-CB149 also dose-dependently increased the EC_{50} of dexamethasone, indicating a competitive antagonism (Fig. 4B). However, maximum induction by dexamethasone was affected at 5 µM, showing a mixed type of antagonism at higher exposure levels. 3-$MeSO_2$-DDE showed a high toxicity at 10 µM, implying that toxicity is probably the cause of the noncompetitive antagonism seen at 5 µM (data not shown). 3-$MeSO_2$-CB101 (0.5–5 µM) did not antagonize the effect of dexamethasone.

The binding of 3-$MeSO_2$-CB149 to GR led us to screen 24 methylsulfonyl PCBs for their affinity to GR in mouse liver cytosol. As shown in Table 1, several methylsulfonyl PCBs bound to GR with a higher affinity than did 3-$MeSO_2$-CB149. The effect of one of the potent methyl sulfones, 4-$MeSO_2$-CB149 (IC_{50} 15 µM; see Fig. 2 for structure), is shown in Figure 5 together with effects of 3-$MeSO_2$-CB149 (IC_{50} >30 µM), the endogenous substrate corticosterone (IC_{50} 40 nM), and unlabeled dexamethasone (IC_{50} 10 nM). 3-$MeSO_2$-DDE did not compete with dexamethasone for binding to mouse GR (data not shown).

Discussion

The present study shows that the PCB methyl sulfone 3-$MeSO_2$-CB149 has an affinity to human GR and appears to competitively antagonize the effects of the endogenous hormone, similarly to the drug RU486. When comparing 3-$MeSO_2$-CB149 with RU486, the relative affinity of 3-$MeSO_2$-CB149 to GR (IC_{50} = 1 µM) is almost 1,000-fold less than that of RU486 (data not shown). Still, 3-$MeSO_2$-CB149 was active in GRAF cells at a concentration of 500 nM. The inhibitory effect of 2–5 µM 3-$MeSO_2$-CB149 on dexamethasone-dependent ALP induction in cells was comparable to that of 10 nM RU486, indicating that the potency in cells of 3-$MeSO_2$-CB149 is 200–500 times less than that of RU486. However, 3-$MeSO_2$-CB149 may not be the most potent PCB methyl sulfone, as indicated by a fourfold higher affinity of 4-$MeSO_2$-CB149 than of 3-$MeSO_2$-CB149 to mouse GR. Considering the high *in vivo* persistency of 3-$MeSO_2$-CB149, in contrast to the fairly rapid metabolism of RU486 ($t_{1/2}$ = 20 hr in man) (*15*) we suspect that the difference in biological potency between these compounds *in vivo* is less than that observed *in vitro*.

IC_{50} values of 1 µM for the binding of 3-$MeSO_2$-CB149 to human GR (cellular extract) and of 15 µM for the binding of 4-$MeSO_2$-CB149 to mouse GR (liver cytosol) may not seem impressive in comparison with IC_{50} values of drugs such as RU486. However, these values are comparable to IC_{50} values reported for the interaction between other xenobiotics and the sex hormone receptors (*16,17*).

The difference in IC_{50} values between GRAF cells and mouse liver cytosol may, to some extent, be caused by species differences. Another plausible explanation is the higher protein concentration in the liver cytosol than in the GRAF cell extract. Because lipophilic xenobiotics in an aqueous solution interact with macromolecules, the concentration of free xenobiotics may be lower in the cytosolic fraction than in the GRAF cell extract and, consequently, a higher concentration of xenobiotics may be needed for an equal effect.

The comparison of affinities of 24 methylsulfonyl PCBs to mouse GR enables us to speculate on structure–activity relationships. It seems that only methylsulfonyl PCBs with three chlorines in *ortho*-positions (2,6,2′) bind to GR. As judged from IC_{25} values of the 4 pairs of 3- and 4-substituted methylsulfonyl PCBs, a methylsulfonyl group in the 4-position relative to the 3-position increases the affinity roughly by a factor of two. Because all these compounds also have a chlorine in the 4′-position, this appears to be an important structural determinant for binding. Thus, it seems that a methylsulfonyl PCB should contain three *ortho*-chlorines and be as extended as possible, with a 4′-chlorine on one end and a methylsulfonyl group on the other 4-position for maximal binding affinity to mouse GR.

Methyl sulfone metabolites of PCBs are as widespread and persistent as the parent PCBs (*18*), making them a potential hazard to humans and animals worldwide. For example, 16 different PCB methyl sulfones and 1 DDE methyl sulfone are present in mother's milk in Sweden, with 4-$MeSO_2$-CB149 being the most abundant and 3-$MeSO_2$-CB149 being the third most abundant methyl sulfone. The concentration of these two compounds in pooled milk was 0.6 ng/g (lipid weight) in

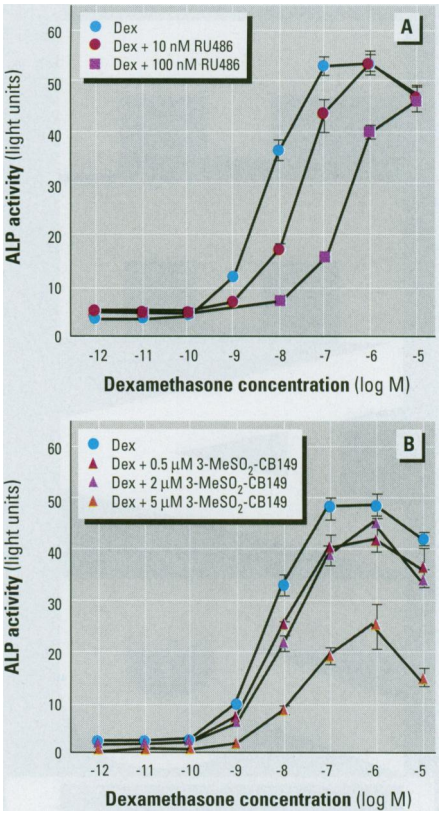

Figure 4. Effect of (A) RU486 and (B) 3-CB149 on the induction of alkaline phosphatase (ALP) in GRAF cells by increasing concentrations of dexamethasone (Dex). Effects on cell morphology were observed in cells exposed to 5 µM 3-$MeSO_2$-CB149. The concentration of 10^{-12} M in the graph represents absence of dexamethasone. Each point represents mean ± standard deviation of triplicate samples.

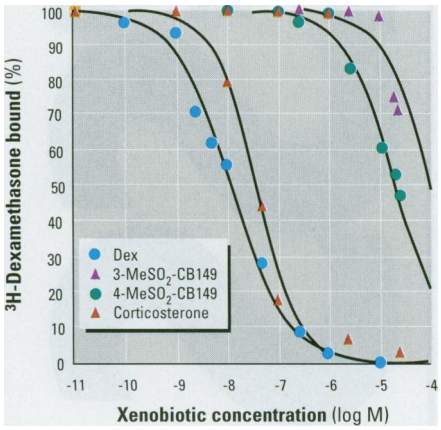

Figure 5. Competition binding studies to the mouse glucocorticoid receptor (GR). Receptor (1 nmol) was incubated overnight with ^{3}H-dexamethasone and either 3-$MeSO_2$-CB149, 4-$MeSO_2$-CB149, corticosterone, or unlabeled dexamethasone (Dex) after which ^{3}H-dexamethasone bound to GR was quantified. Each point represents mean of duplicate samples.

most abundant and 3-MeSO$_2$-CB149 being the third most abundant methyl sulfone. The concentration of these two compounds in pooled milk was 0.6 ng/g (lipid weight) in 1992 (*19*). Much higher concentrations of PCB methyl sulfones have been found, for example, in Baltic seals (*20*), suggesting that some species are more subject to biological effects of PCB methyl sulfones than humans. Total PCB methyl sulfone concentrations of 110 µg/g (lipid weight) have been found in the blubber of gray seals, where 3-MeSO$_2$-CB149 is the most abundant (*5*). The PCB methyl sulfones accumulate in liver relative to blubber in gray seals (*5*); therefore, these animals may be exposed to even higher concentrations of PCB methyl sulfones. The disease syndrome in Baltic seals (*4*) indicates an interference with the glucocorticoid homeostasis. There are also other indications of environmental pollutants acting on the glucocorticoid system. Field studies showed a reduced ability of fish from polluted waters to elevate cortisol levels in response to acute stress (*21,22*). Likewise, DDT and corticosterone induced a similar pathological development in amphibians, which was interpreted as a potential corticosterone-mimicking action of DDT (*23*).

In conclusion, several PCB methyl sulfones have affinity to GR, which may affect the glucocorticoid homeostasis. Therefore, the concern for endocrine disruptors should also be directed towards the glucocorticoid system.

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